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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

ATR is protein kinase required for both DNA damage-induced cell cycle checkpoint responses and the DNA replication checkpoint, which prevents mitosis before completion of DNA synthesis. Although ATM and ATR share many substrates, the different phenotypes observed in ATM- and ATR- deficient mice indicate these kinases are not functionally redundant. We have shown that ATR but not ATM phosphorylated human Rad17 on serines 635 and 645 in vitro and in vivo. In undamaged cells, these same sites are phosphorylated in late G1, S, and G2, but not in early G1; however, treatment with genotoxic agents induces phosphorylation of hRad17 in early G1. Additionally, Rad17 is phosphorylated in actively replicating but not quiescent cells in an ATM-independent manner. Expression of a phosphorylation mutant hRad17, with both serines changed to alanine, abolishes IR-induced activation of the G1/S checkpoint and results in cellular sensitivity to IR in the breast cancer cell line, MCF-7. These results suggest ATR and hRad17 are required for an ATM-independent DNA damage response pathway. Additionally, these results demonstrate the kinase activity of ATR on hRad17 is activated in response to DNA damage and during normal replication and indicates the hRad17 may play a role during normal DNA synthesis.

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Introduction:

Upon DNA damage and replication block, eukaryotic cells activate evolutionally conserved checkpoint-signaling pathways, which in turn activate cell cycle checkpoints (3, 22). Loss of these checkpoints results in genomic instability and cancer predisposition. In fission yeast, six evolutionally conserved checkpoint Rad proteins, Rad17, Rad3, Rad26, Rad9, Rad1, and Hus1 are required for replication and DNA damage induced checkpoints; and each is required for the phosphorylation and activation of the downstream effector kinases, Chk1 and Cds1 (3, 11, 13-15). Human homologues to these checkpoint Rad proteins have been identified: Rad17 (hRad17), Rad3 (ATR), Rad26 (ATRIP), Rad9 (Rad9), Rad1 (Rad1), and Hus (Hus1) and these proteins are also required for checkpoint activation through human Chk1 (2, 5, 20, 21, 23). In response to DNA damage and replication block, hRad17, which shares significant homology to each of the five RFC subunits and forms a pentimeric clamp-loading complex with the four small RFC subunits, is phosphorylated on Ser^{635} and Ser^{645} by ATR (1, 16).

Since hRad17 is phosphorylated by ATR and fission yeast Rad17 is required for activation of Cds1, it is possible that the phosphorylation of hRad17 may be required for Chk2 activation. Chk2 is required for checkpoint activation through its phosphorylation of Cdc25 and BRCA1, respectively (10, 12). In response to DNA damage, Chk2 and ATR phosphorylate BRCA1 on Ser^{988} and Ser^{1423}, respectively (10, 19). These data suggest the human checkpoint Rad proteins are involved in a signaling cascade that is required for the activation and phosphorylation of BRCA1. The goal of this work is to delineate the mechanism of checkpoint activation between the checkpoint Rad proteins ATR and hRad17 and subsequently their potential downstream targets Chk2 and BRCA1.

Body:

Task 1. To determine the functional significance of ATR/ATM mediated phosphorylation of hRad17 in cell cycle checkpoints.

A. Delineate the kinase/substrate relationship between ATR/ATM and hRad17 and map ATR phosphorylation sites of hRad17 using both *in vitro* and *in vivo* approaches.
B. Analyze hRad17 phosphorylation during the cell cycle.
C. Investigate the biological significance of ATR mediated phosphorylation of hRad17 in replication and DNA damage induced checkpoint control.

A. **Delineate the kinase/substrate relationship between ATR/ATM and hRad17 and map ATR phosphorylation sites of hRad17 using both *in vitro* and *in vivo* approaches.**

To examine whether hRad17 is a substrate of ATR and/or ATM, *in vitro* kinase assays were performed. Immunoprecipitated ATR, but not ATM, phosphorylated glutathione S-transferase (GST)-full length hRad17 (Fig. 1A, top two panels, lanes 3 & 6). The immunoprecipitated ATM was active, as it phosphorylated known substrates, GST-N-p53$^{1-106}$ (9)and GST-hRad9$^{255-295}$ (4) (Fig. 1A, bottom two panels, and lanes 4 and 5). ATR also phosphorylated p53 efficiently but did not phosphorylate GST-hRad9$^{255-295}$, an ATM specific substrate (Fig. 1A, bottom two panels, and lanes 1 and 2). The kinase/substrate relationship between ATR and hRad17 was further confirmed by using recombinant wild type and kinase inactive ATR (Flag-ATR^{Wt} and Flag-ATR^{Ki}), only Flag-ATR^{Wt} phosphorylated GST-full length hRad17 (Fig. 1B). These results differentiate ATR and ATM substrate specificity *in vitro*.

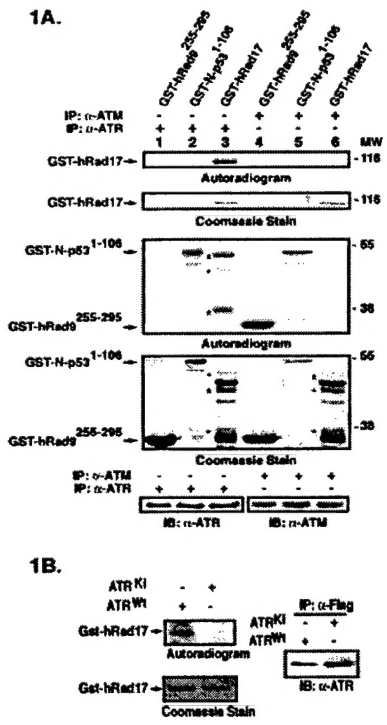


Figure 1. Phosphorylation of hRad17 by ATR *in vitro*. (A) Kinase assays using immunoprecipitated ATM and ATR. GST-hRad17 was incubated with ATM (top two panels, lane 6) and ATR (top two panels, lane 3) immunoprecipitated from HeLa cells treated with 10 Gy IR. GST-N-p53$^{1-106}$, a known substrate of the two kinases was incubated with ATM (bottom two panels, lane 5) or ATR (bottom two panels, lane 2). GST-hRad9$^{255-295}$, a known substrate of ATM was incubated with ATM (bottom two panels, lane 4) or ATR (bottom two panels, lane 1). Three μg of substrate was used in each reaction. The kinase reaction products were separated by SDS-PAGE and analyzed by Coomassie staining and autoradiograph. Levels of ATR and ATM in the kinase reactions were determined by immunoprecipitation followed by western blotting. (B) Kinase assays using recombinant ATR protein. Human kidney 293 cells were transiently transfected with Flag-tagged wild-type (ATR^{Wt}) or kinase-inactive mutant ATR (ATR^{Ki}). Cells were treated with 10 Gy IR 36 h post-transfection and lysed 1 h post-IR. GST-hRad17 fusion proteins were incubated with recombinant ATR immunoprecipitated with α-Flag antibodies. Immunoprecipitation with α-Flag antibodies followed by immunoblotting with α-ATR antibodies confirmed the presence of recombinant ATR.

Two consensus ATR/ATM phosphorylation sites, Ser^{635} and Ser^{645}, and a non-preferred SQ site, Ser^{180}, are present in hRad17 (Fig. 2A). GST-full length hRad17^{Wt} and GST-hRad17^{S180A} were readily phosphorylated by ATR. However, substitution of Ser^{635} and Ser^{645} to alanine greatly reduced but did not completely abolish ATR-mediated phosphorylation of hRad17 (Fig. 2B). Taken together, these data demonstrated that GST-hRad17 is mainly phosphorylated on Ser^{635} and Ser^{645} by ATR *in vitro* but additional site may exist in hRad17.

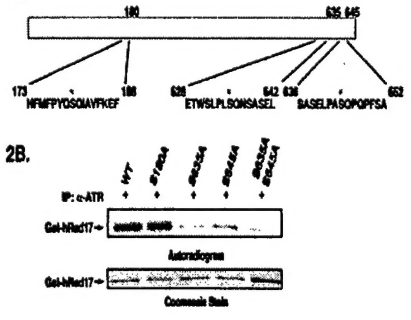


Figure 2. Phosphorylation of hRad17 on Ser^{635} and Ser^{645} *in vitro* (A) Schematic representation of mutant hRad17 proteins. Site-specific mutation of Ser to Ala was confirmed by DNA sequencing. (B) Ser^{635} and Ser^{645} of hRad17 are substrate sites of ATR *in vitro*. Wild type and mutant hRad17 fusion proteins were incubated with ATR and the resultant proteins were analyzed as described in (Figure 1A).

To confirm that Ser^{635} and Ser^{645} of hRad17 are phosphorylated *in vivo*, phosphospecific antibodies against KLH-conjugated ETWSLPLS(PO$_3$)QNSASEL and SASELPAS(PO$_3$)QPQPFSA peptides were generated and their specificity tested using GST-hRad17. The antibodies react specifically with GST-hRad17 that had been incubated with immunoprecipitated ATR (Fig. 3, lanes 1 and

4) but not with purified GST-hRad17 or GST-hRad17 incubated with immunoprecipitated ATM (Fig. 3, lanes 2 & 3 and 5 & 6).

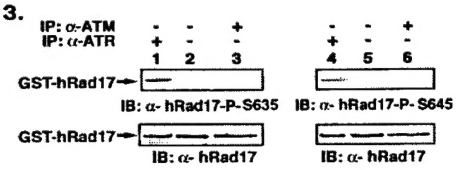


Figure 3. Specificity of the α-hRad17 phosphospecific antibodies. GST-hRad17 was incubated with ATR (top two panels, lane 1 & 4) or ATM (top two panels, lane 3 & 6) immunoprecipitated from HeLa cells and immunoblotting was with phosphopeptide antibodies.

We next examined whether phosphorylation of both sites of endogenous hRad17 is stimulated by various treatment with genotoxic agents. There were basal levels of Ser^{635} and Ser^{645} phosphorylation in untreated asynchronous human fibroblast VA-13 cells and treating cells with hydroxyurea, aphidicolin, IR, or UV-irradiation all resulted in elevated phosphorylation of endogenous hRad17 (Fig. 4). Levels of hRad17 and β–actin remained constant in the untreated or treated cells.

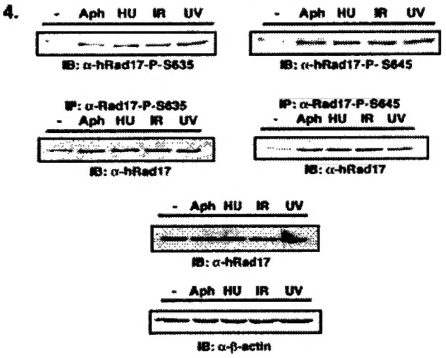
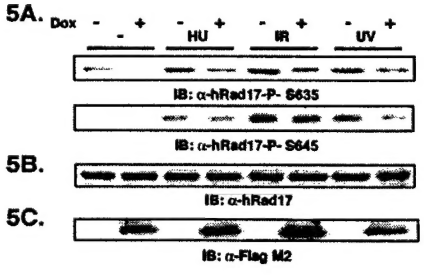


Figure 4. Phosphorylation of hRad17 on Ser^{635} and Ser^{645} *in vivo*. Human fibroblast VA-13 cells were mock-treated, treated with 5μg/ml aphidicolin for 20 h, 1mM hydroxyurea for 24 h, 10 Gy IR, or 50J/m^2 UV irradiation. Cells lysates were subjected to SDS-PAGE and immunoblotting was performed using the indicated antibodies or lysates were immunoprecipitated with α-hRad17-P-^{S635} or α-hRad17-P-^{S645} antibodies. Immunoblotting of the immunoprecipitated lysates were performed using α-hRad17 antibody, 31E9. Western blotting analysis of hRad17 protein in the whole cell extracts. Levels of hRad17 and β–actin remain constant in untreated and treated cells.

We next wanted to study what kinase was responsible for the phosphorylation of hRad17 *in vivo*. To this end, we studied the phosphorylation of Ser^{635} and Ser^{645} in cells expressing kinase-inactive ATR under the regulation of tetracycline. Upon induction of ATR^{Ki} phosphorylation of Ser^{635} and Ser^{645} were reduced two to ten fold, in untreated cells and cells under genotoxic stress, based on densitometric analysis (Fig. 5A and data not shown). Protein levels of hRad17 did not change in response to DNA damage, replication block, or doxycycline treatment (Fig. 5B). Expression of ATR^{Ki} was also similar in untreated and treated cells (Fig. 5C). As previously reported(18), the residual phosphorylation in cells treated with doxycycline is likely due to the remaining endogenous ATR activities.

Figure 5. ATR-dependent phosphorylation of Ser^{635} and Ser^{645} of hRad17. (A) Analysis of Ser^{635} and Ser^{645} phosphorylation in cells expressing ATR^{Ki}. Cells expressing ATR^{Ki} under tetracycline regulation were grown in the presence of doxycycline for 72 h. Soluble proteins were prepared and cell extracts were separated by SDS-PAGE. Immunoblotting was performed using indicated antibodies. (B) Immunoblotting analysis of hRad17 before and after DNA damage and replication block. Soluble proteins from treated and untreated cells were subjected to SDS-PAGE and immunoblotted with α-hRad17 antibody 31E9. (C) Immunoblotting analysis of recombinant ATR^{Ki} expression. Expression of ATR^{Ki} was determined by SDS-PAGE followed by immunoblotting with α-Flag-M2.



To test whether ATM is required for phosphorylation of hRad17 Ser^{635} and Ser^{645} *in vivo*, we analyzed the phosphorylation events using extracts from EBS (ATM-deficient cells) and YZ5 (ATM-complemented

cells) prepared from mock- or 30 Gy IR-treatment at indicated time points (Fig. 6). Phosphorylation on Ser^{635} of hRad17 was induced 1.61, 1.91, and 1.81 fold in response to DNA damage at 1, 2, and 4 hours, respectively, in ATM-deficient cells. Phosphorylation on the same serine in ATM-deficient cells expressing recombinant ATM was induced 1.76, 1.76, and 2.46 fold at the same time points. Phosphorylation on Ser^{645} of hRad17 was induced 1.87, 1.72, and 1.60 fold in response to DNA damage at 1, 2, and 4 hours, respectively, in ATM-deficient cells. Phosphorylation on the same serine in ATM-deficient cells expressing recombinant ATM was induced 1.7, 2.57, and 3.31 fold at the same time points. The fold induction in ATM-deficient cells at 4 hr post-IR is not as apparent, which may be relevant to the higher basal phosphorylation seen in these cells in the absence of DNA damage. These data suggest that ATR, but not ATM, is likely to be the kinase responsible for phosphorylating Ser^{635} and Ser^{645} of hRad17 in proliferating cells and in cells under genotoxic stress.

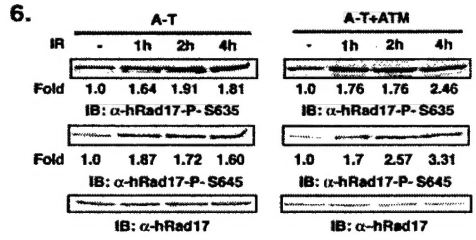


Figure 6. ATM-independent phosphorylation of Ser^{635} and Ser^{645} of hRad17. EBS and YZ5 cells were mock-treated or treated with 30 Gy IR and harvested 1, 2 or 4 h post-treatment. Bottom panel, western blotting analysis of hRad17 in the whole cell extract.

B. Analyze hRad17 phosphorylation during the cell cycle.

Since basal levels of Ser^{635} and Ser^{645} phosphorylation were detected in asynchronous cell populations without DNA damage (Fig. 4 and 5), we examined whether there are cell cycle-regulated modification of these residues. T24 cells were density arrested, released, and harvested at specific phases of the cell cycle. Ser^{635} and Ser^{645} became phosphorylated at the start of S-phase and phosphorylation continued throughout the remainder of the cell cycle (Fig. 7A). Protein levels of hRad17 remained constant throughout the cell cycle (Fig. 7A). We next determined if DNA damage-induced phosphorylation of these residues occurs in a cell cycle-dependent manner. Phosphorylated Ser^{635} and Ser^{645} were readily detectable in T24 cells in the G1 phase (G11) upon exposure to IR but not in mock-treated cells (Fig. 7B). Similar results were obtained in response to UV treatment (data not shown). In contrast to cells in the G1 phase, levels of phosphorylation of Ser^{635} and Ser^{645} were not substantially enhanced during mid-S (G24) and G2 phases (G33) in response to IR. The cell cycle distribution was confirmed by FACS analysis, showing approximately 90% of cells were in G1 (G11), 60% in S (G24), and 60% in G2 (G33) respectively.

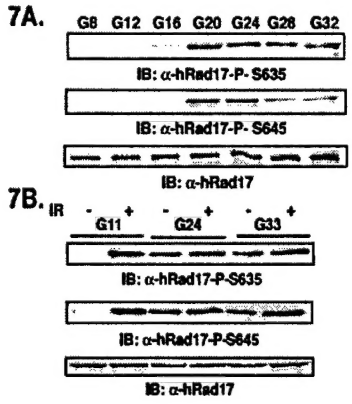


Figure 7. Cell cycle-dependent phosphorylation of Ser^{635} and Ser^{645} of hRad17. (A) Immunoblot analysis of phosphorylation at Ser^{635} and Ser^{645} of hRad17 during the cell cycle. Density arrested T24 cells were released and harvested at indicated time points G8, G12, G16, G24, G28, and G33 representing 8 h, 12 h, etc. after density release, respectively. (B) Immunoblot analysis of hRad17 phosphorylation during different cell cycle phases. Density arrested T24 cells were released for 11 h, 24 h, and 33 h and harvested 1 h post-treatment.

To demonstrate that these cell cycle dependent phosphorylation events are not caused by the synchronization protocol, we examined the phosphorylation status of murine Rad17 in replicating and non-replicating tissues. Phosphorylation on both Ser^{647} and Ser^{657} residues of *Mm*Rad17, corresponding to hRad17 Ser^{635} and Ser^{645}, was observed in extracts from both undamaged and IR-treated testis (Figure 8A). In contrast to this replicating tissue, the same two residues were phosphorylated only after DNA damage in quiescent lung tissue (Figure 8A). Since the kinase activity of ATR increases in response to S-phase (8) and ATM and ATR are both involved in the activation of S-phase dependent checkpoints (7, 17), we asked whether ATM influences the phosphorylation of mammalian Rad17 in replicating tissues after IR. Interestingly, the absence of ATM had little effect on the phosphorylation of *Mm*Rad17 in the presence or absence of DNA damage in testis (Figure 8B). These results indicate that mammalian Rad17 is normally phosphorylated during replication in an ATM-independent manner and suggests that hRad17 may play a role during DNA replication in unperturbed cells.

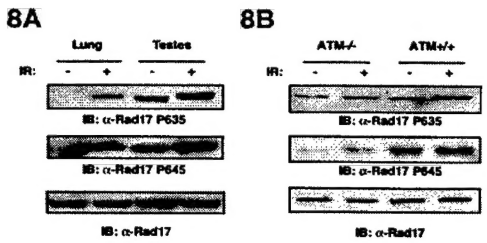


Figure 8. Phosphorylation of *Mm*Rad17 in replicating and quiescent mouse tissues. A. Phosphorylation of *Mm*Rad17 in testis and lung tissues. Mice were mock-treated or treated with 10 Gy IR and sacrificed 1 h post-treatment and lysates were prepared for western blotting analysis. B. Phosphorylation of *Mm*Rad17 in $Atm^{-/-}$ mice. Mice were treated as in A, and lysates were prepared for western blotting analysis.

C. Investigate the biological significance of ATR mediated phosphorylation of hRad17 in replication and DNA damage induced checkpoint control.

In subsequent experiments, we determined whether phosphorylation of hRad17 on Ser^{635} and Ser^{645} is required for G1/S checkpoint activation. Similar levels of recombinant wild type and mutant HA-hRad17 were detected in cells transfected with pcDNA3.1-HA-$hRad17^{WT}$ or pcDNA3.1-HA-$hRad17^{S635A/S645A}$ (Fig. 9A). Both recombinant wild type and mutant HA-hRad17 interacted with p37/RFC (Fig 9B). Additionally, unphosphorylated hRad17 from undamaged G1 synchronized cells and phosphorylated hRad17 from damaged G1 synchronized cells interacted with p37/RFC (Fig 9B), suggesting that phosphorylation of hRad17 is not required for the CLC formation and that the four small RFC subunits form a stable complex as seen in yeast (6).

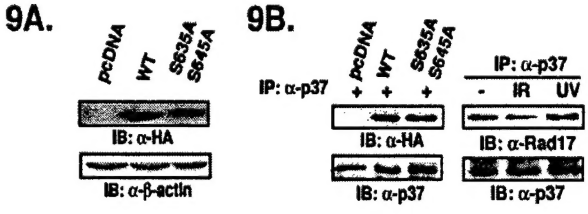


Figure 9. Effects of expression of $hRad17^{S635A}$ and $hRad17^{S645A}$ on G1/S checkpoint activation. (A) Immunoblotting analysis of recombinant HA-hRad17 expression in the transfected cells. Proteins in the lysates were separated by SDS-PAGE followed by immunoblotting analysis using α-HA antibody. (B) Recombinant HA-hRad17 and endogenous hRad17 interact with p37/RFC. Immunoprecipitation was carried out using α-p37/RFC antibodies and western blotting was with antibodies as indicated.

The effects of Ser^{635} and Ser^{645} phosphorylation on G1/S checkpoint were assessed by co-transfecting pcDNA3.1, pcDNA3.1-HA-$hRad17^{WT}$, or pcDNA3.1-HA-$hRad17^{S635A/S645A}$ and pEGFP at a 10:1 ratio into MCF-7 cells, which express wild-type p53. Overexpression of $hRad17^{S635A/S645A}$ but not vector or wild-type hRad17 (Fig. 10) abolished IR-induced G1/S checkpoint activation, suggesting phosphorylation of Ser^{635}

and Ser^{645} of hRad17 is a critical event required for checkpoint activation following DNA damage (Fig. 10).

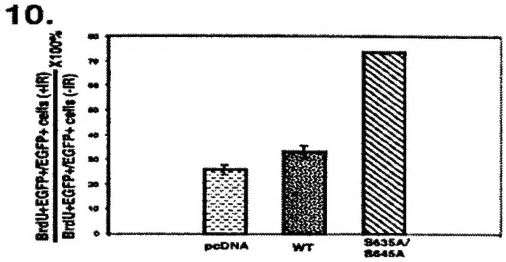


Figure 10. Abberent G1/S checkpoint activation upon IR in cells overexpressing phosphomutant hRad17. The ratio of BrdU- and EGFP-double positive cells to EGFP-positive cells was determined in mock- and IR-treated cells, respectively. At least 350 cells were counted from each plate. The mean and s.d. were calculated from three separate plates.

Task 2. Determine if phosphorylation of hRad17 is required for BRCA1 Ser^{988} and Ser^{1423} phosphorylation.

A. Generate a stable tetracycline inducible MCF-7 cell line expressing wild-type and phospho-mutant hRad17.
B. Perform assays to determine if Chk2 is phosphorylated and activated in these cells in response to genotoxic stress.
C. Perform assays to determine if BRCA1 is phosphorylated in these cells in response to genotoxic stress.
D. Determine if Rad17 is upstream of BRCA1 by using BRCA1 Δexon11/Δexon11 MEFs.

A. Generate a stable tetracycline inducible MCF-7 cell line expressing wild-type and phospho-mutant hRad17.

In response to DNA damage, we know that phosphorylated hRad17 plays a role in checkpoint activation and that yeast Rad17 is required for activation of Cds1 the yeast homology of human Chk2. Therefore, we want to determine if phosphorylation of hRad17 by ATR is required for Chk2 activation and thereby regulate the phosphorylation of BRCA1. To accomplish this, we generated a stable tetracycline inducible MCF-7 cell line expressing HA-tagged wild-type or phosphomutant hRad17. Levels of recombinant HA-hRad17 are at least 3 fold higher than endogenous hRad17 (Figure 11A). Treating cells, which are overexpressing phosphomutant hRad17, with IR results in loss of cell viability (Figure 11B). These results coupled with our previous data, (Figure 10) suggest the cellular sensitivity to the IR may result from the loss of the G1/S checkpoint.

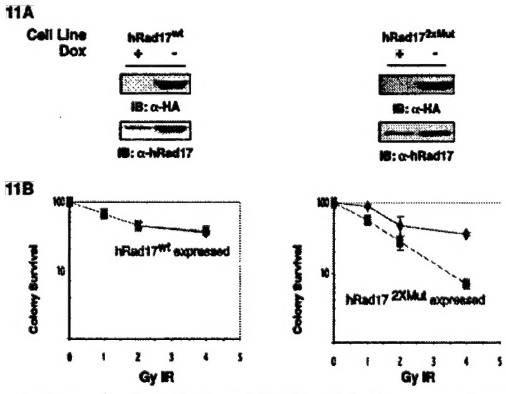


Figure 11. Characterization of MCF-7 wild type and phosphomutant hRad17 cell lines. (A) Western blot analysis of MCF-7 inducible cell lines. Cells were grown in the presence of 1ug/ml doxycycline for 72 h then harvested. Protein expression was determined by western blotting analysis. (B) Cells expressing phospho-mutant hRad17 are sensitive to DNA damage. Colony survival assays were performed by treating equal numbers of cells to 1, 2, or 4 Gy IR. Colonies were cultured in doxycycline free or supplemented media for 21 days and then counted.

B. **Perform assays to determine if Chk2 is phosphorylated and activated in these cells in response to genotoxic stress.**
C. **Perform assays to determine if BRCA1 is phosphorylated in these cells in response to genotoxic stress.**
D. **Determine if Rad17 is upstream of BRCA1 by using BRCA1 Δexon11/Δexon11 MEFs.**

Currently, we are investigating these remaining three aims.

Key Research Accomplishments:

- ATR but not ATM phosphorylates hRad17 *in vitro* and *in vivo*.
- hRad17 is phosphorylated during S-phase in unperturbed cells.
- Rad17 is phosphorylated in undamaged replicating tissues.
- Phosphorylation of hRad17 is not required for the formation of the RFC-like clamp-loading complex.
- Phosphorylation of hRad17 is required for checkpoint activation in response to DNA damage.

Reportable Outcomes:

Development of Cell lines:

Generated tetracycline inducible MCF-7 cell line.
Generated tetracycline inducible MCF-7 cell line expressing wild type and phosphomutant HA-tagged hRad17.

Manuscripts:

Post S, Weng YC, Cimprich K, Chen LB, Xu Y, Lee E. Phosphorylation of serines 635 and 645 of human Rad17 is cell cycle regulated and is required for G1/S checkpoint activation in response to DNA damage. *Proc. Natl. Acad. Sci. U S A.* 2001 Nov 6; **98** (23): 13102-13107.

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Conclusions:

Understanding how hRad17 regulates and thereby activates the checkpoint-signaling cascade in response to DNA damage is critical to understanding how cells respond to DNA damage. To date, our laboratory, as well as others, has shown that the phosphorylation of hRad17 by ATR is required for checkpoint activation in response DNA damage. While these findings are important, the most intriguing data is that hRad17 also becomes phosphorylated on these same serine residues at the start of S-

phase. This suggests that hRad17 may play a role during normal DNA synthesis. Given these new findings and the fact that some tumors overexpress hRad17, it is interesting to speculate that hRad17 may be involved in and may be required for the regulation of DNA replication in actively growing cells. If this is true, then hRad17 becomes an attractive " target protein" for therapeutic research, since it is overexpressed in cancerous cells and undergoes post-translational modifications (phosphorylation) in replicating cells.

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